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Wysok, Beata

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Pathogenicity of *Campylobacter* strains of poultry and human origin from Poland

Beata Wysok[a], Joanna Wojtacka[a], Rauni Kivistö[b,*]

[a] Department of Veterinary Public Health, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Oczapowskiego 14, 10-917 Olsztyn, Poland
[b] Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Agnes Sjöbergin katu 2, 00014 Helsinki, Finland

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ABSTRACT

The aim of this study was to determine the pathogenic markers associated with *Campylobacter* infection in humans. A total of 104 *Campylobacter* isolates obtained from poultry and humans were examined for the presence of nine virulence genes and their ability to adhere to, invade and produce cytotoxin were defined using HeLa cells. The diversity of the *Campylobacter* spp. isolates was studied based on sequencing of the SVR-region of *flaA* gene. Altogether 45 *flaA*-SVR alleles were identified among 104 *Campylobacter* isolates of poultry and human origin. All *Campylobacter* isolates possessed *flaA*, *cadF* and *racR* genes involved in adherence. Accordingly, all poultry and human isolates exhibited adherence towards HeLa cells at mean levels of 0.95% and 0.82% of starting viable inoculum, respectively. The genes involved in invasion (*iam* and *pldA*) and cytotoxin production (*cdtA*, *cdtB* and *cdtC*) were also widely distributed among the human and poultry *Campylobacter* isolates. Significantly higher invasiveness was observed for poultry isolates (mean level of 0.002% of starting bacterial inoculum) compared to human isolates (0.0005%). Interestingly the *iam* gene, associated with invasion, was more common in human (100%) than poultry (84%) isolates, and the poultry isolates lacking the *iam* gene showed a marked reduction in their ability to invade HeLa cells. Moreover, *virB11* was present in 22% of the poultry and 70.4% of the human isolates. Strains lacking *virB11* showed a slight reduction in invasion, however in the absence of *iam* even the poultry isolates containing *virB11* were unable to invade HeLa cells. The mean cytotoxicity of *Campylobacter* isolates from poultry and human was 26.7% and 38.7%, respectively. Strains missing both the *cdtB* and *cdtC* genes were non-cytotoxic compared to strains containing all three *cdtABC* genes, which were the most cytotoxic among the *C. jejuni* and *C. coli* isolates from both sources. No cytotoxic effect was observed in only 4% of poultry and 5.6% of human isolates.

1. Introduction

The widespread occurrence of pathogens in the animal population contributes to their frequent presence in food and leads to foodborne diseases in human. According to the scientific report of the European Food Safety Authority (EFSA, 2019), *Campylobacter* has been the most frequently isolated gastrointestinal bacterial pathogen in humans for the last 10 years. In the EU in 2018 *Campylobacter* infection was confirmed in 246,571 cases, with an incidence of 64.1 per 100,000 population. However, the Centers for Disease Control and Prevention (CDC) estimates, that for every *Campylobacter* case reported, there are thirty non-diagnosed cases.

Poultry and poultry products are considered to be the most important foodborne sources of campylobacteriosis in humans. According to data published by EFSA (2010), *Campylobacter* was found in 31.3% of fresh broiler meat samples. Simultaneously, in four EU countries, including Poland, the isolation rate of this pathogen exceeded 50% in broilers. These data are in accordance with studies recently conducted in Poland (Wieczorek and Osek, 2015), Spain (Perez-Arnedo and Gonzalez-Fandos, 2019) and China (Zhang et al., 2018) describing poultry as a common reservoir of *Campylobacter* spp. Infection in humans is most likely due to the consumption of raw or not thoroughly cooked poultry meat. However, the *Campylobacter* infections may also develop as a result of cross contamination of ready-to-eat foods. Also lack of hygiene may lead to the transfer of bacteria from hand to mouth during food preparation. The infection in humans is relatively frequent since the infectious dose is as low as 500 to 800 cfu (Janssen et al., 2008; Kaakoush et al., 2015). In most cases, symptoms occur 24 to 72 h following oral exposure and last up to 7 days (Kaakoush et al., 2015). Infected people exhibit a range of symptoms varying from mild watery

* Corresponding author at: University of Helsinki, Faculty of Veterinary Medicine, Agnes Sjöbergin katu 2, 00014 Helsinki, Finland.
E-mail address: rauni.kivisto@helsinki.fi (R. Kivistö).

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to severe inflammatory diarrhea with blood and leukocytes (Janssen et al., 2008).

The course of campylobacteriosis is affected both by the individual hosts sensitivity and bacterial factors. The genome of *Campylobacter* contains many virulence genes associated with motility, adherence and colonization of the intestinal mucosa, invasion and toxin production (Friis et al., 2005; Redondo et al., 2019). Bacterial adhesion and invasion are well-established early events before the initiation of inflammatory processes and diarrheal development (Bhavsar and Kapadnis, 2006). However Van Vliet and Ketley (2001) underlined, that the ability of bacteria to invade the host cells, despite the cytopathic effects on epithelial cells, very often isn't sufficient as the only factor responsible for developing infection. Therefore, it is important to conduct studies determining the toxic effect of *Campylobacter*. Due to the high diversity of *Campylobacter* spp. the pathogenic mechanisms causing clinical symptoms are still not very well understood (Redondo et al., 2019). Moreover González-Hein et al. (2013) underlined, that although many genes related to the pathogenicity of *Campylobacter* have been reported, the relationships between these genes and the sources of strains are not clear. Both *in vitro* and *in vivo* tests can be used to determine the pathogenicity of bacteria. According to Friis et al. (2005) *in vitro* cell culture methods are useful in determining the host – bacterial interactions during *Campylobacter* infection. These methods are more suitable than biological tests because they are more affordable, easy to standardize and ethically approvable. Among available cell lines, HeLa cells have been widely used for studies of pathogenic properties of many bacterial enteric infections (Gilbert and Slavik, 2004; Morais-Rios et al., 2018).

Various subtyping methods may be used in order to track *Campylobacter* spread among different sources. For example, *flaA* sequencing has been highly efficient in describing the diversity among *Campylobacter* populations (Marotta et al., 2015; Wieczorek et al., 2019). The entire coding sequence of the *flaA* gene (1764 nucleotides) contains two highly variable regions, one from base position 700 to 1450 and the short variable region (SVR) from base position 450 to 600 (Meinersmann et al., 1997). Ghorbanalizadgan et al. (2016) noticed that the results obtained by assessing the level of discrimination that could be discerned from the entire sequence of the total *flaA* gene and only the *flaA*-SVR were very similar. Thus, the sequence of the *flaA*-SVR is more commonly used for genotyping *Campylobacter* isolates (Aslantas, 2019; Giacomelli et al., 2012; Wassenaar et al., 2009;). It has been shown to be useful for discriminating between closely related *Campylobacter* strains (Hiett et al., 2002). Meinersmann et al. (1997) confirmed the utility of DNA sequence analyses of the SVR segment for the characterization of *Campylobacter* isolates for epidemiologic studies. The variation in *flaA* indexed by SVR typing is under diversifying selection, thereby making it a suitable target for short-term epidemic investigations (Dingle et al., 2002), what was relevant in our study to estimate the transmission of *Campylobacter* isolates between different sources.

The present study was undertaken to determine virulence properties of *Campylobacter* strains isolated from poultry carcasses and humans feces. The genetic diversity of the isolates was assessed using *flaA*-SVR sequencing. Moreover, the incidence of genes associated with virulence, more specifically cytotoxin production, ability to adhere to and invade epithelial cells and the expression of the respective activities *in vitro* using HeLa cells were explored.

2. Materials and methods

2.1. Isolation and identification of bacterial strains

To obtain equivalent numbers of isolates from each source the number of tested samples varied between sources. During this study, a total of 80 samples from poultry carcasses and 1413 samples from sporadic cases of human diarrhea were analyzed for the presence of *Campylobacter* spp. All samples were collected from May 2012 to February 2013 from the same district in Poland. The swabs from neck skins of broiler carcasses were taken after chilling in two abbatoirs. The swabs were transfered to sterile 0.1% pepton water and the suspension was transported to the laboratory at 0–5 °C. Next 2 ml of the suspension was transferred to 18 ml of Bolton broth (Oxoid, UK) and the enrichment cultures were grown at 37 °C for 4 h, then at 41.5 °C for 44 ± 4 h under microaerobic conditions (5% O_2, 10% CO_2, and 85% N). Then, a loopful of the suspension was spread on the surface of a charcoal cefoperazone deoxycholate modified agar (mCCDA, Oxoid, UK) and agar Karmali (Oxoid, UK). After incubation under microaerobic conditions for 24–48 h the plates were examined for morphologically typical *Campylobacter* colonies. Single colonies were picked up and confirmed as *Campylobacter* by examination of microscopic morphology, the presence of oxidase activity, motility and lack of microaerobic growth at 25 °C. Subsequently the isolates were subcultured only once in order to minimize changes due to several passages and stored at −80 °C in defibrinated horse blood (Oxoid, UK) with added glycerol (80:20 *v*/v).

The human fecal samples, taken from patients with diarrhea for routine screening of bacterial pathogens, were examined in the regional sanitary-epidemiological units. The stool samples were directly inoculated onto mCCDA and the plates were incubated at 42 °C under microaerobic conditions for 48 h. Further treatment was carried out according to the methodology described above.

Species identification of the isolates was carried out based on a previously described PCR method (Wysok et al., 2015). For this purpose *Campylobacter* isolates cultured on Columbia agar supplemented with blood were suspended in 1 ml of sterile water, and centrifuged at 13000 ×g for 1 min. The precipitate was suspended in Tris buffer. DNA isolation was performed using Genomic - Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. Purity and concentration of the DNA was determined spectrophotometrically.

2.2. Detection of virulence genes

The genomic DNA was amplified by PCR to confirm the presence of genes involved in adherence (*flaA*, *cadF* and *racR*), invasion (*virB11*, *iam* and *pldA*), and responsible for the production of cytolethal distending toxin (*cdtA*, *cdtB* and *cdtC*) by using primers listed in Table 1. Additionally the presence of the *cdtABC* gene cluster in all *Campylobacter* isolates was determined with the primers previously described by Bang et al. (2003). Amplification was performed in a 50 μl reaction mixture containing 5 μl of the PCR buffer (10 – times concentrated), 5 μl of dNTPs (final concentration of 200 μM), 0.5 μl of each primer (final concentration of 0.1 μM), 10 μl $MgCl_2$ (final concentration of 5 mM), 2 μl (2 U) thermostable Taq polymerase (Termo Fisher Scientific, USA), 5 μl of template DNA at the final concentration of 120 ng verified by Nano-Drop™ Spectrophotometer (Thermo Fisher Scientific, USA) and DNase-and RNase-free deionized water. All PCR reactions were carried out using the following conditions: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation for 1 min at 95 °C, annealing at a temperature specific to the primer pair for 1 min and extension for 1 min at 72 °C. The final elongation step was carried out at 72 °C for 5 min. A positive control consisting of DNA extracted from *C. jejuni* ATCC 33291 and *C. coli* ATCC 43478, as well as a negative PCR control consisting of PCR-grade water were included in each PCR run. The PCR product was run on a 2% agarose gel stained with ethidium bromide at a concentration of 5 μg/ml. The size of the amplification product was determined using the 100 bp molecular weight marker.

2.3. *flaA*-SVR sequencing

The DNA of all isolates obtained in this study was subjected for *flaA* short variable region (SVR) PCR and sequencing using primers FLA242FU (5'-CTA TGG ATG AGC AAT T(*AT*)A AAA T-3′) and FLA625RU (5'-CAA G(*AT*)C CTG TTC C(*AT*)A CTG AAG −3′) (Hiett

Table 1
List of primers used in this study.

Target Gene	Sequences (5′ – 3′)	Product size (bp)	Annealing temperature °C	References
16S rRNA for *Campylobacter* spp.	F - ATCTAATGGCTTAACCATTAAAC R - GGACGGTAACTAGTTTAGTATT	857	58	Wieczorek and Osek (2005)
mapA for *C. jejuni*	F - CTATTTTATTTTTGAGTGCTTGTG R - GCTTTATTTGCCATTTGTTTTATTA	589	58	Wieczorek and Osek (2005)
ceuE for *C. coli*	F - AATTGAAAATTGCTCCAACTATG R - TGATTTTATTATTTGTAGCAGCG	462	58	Wieczorek and Osek (2005)
flaA	F - AATAAAAATGCTGATAAAACAGGTG R - TACCGAACCAATGTCTGCTCTGATT	855	53	Datta et al. (2003)
cadF	F - TTGAAGGTAATTTAGATATG R - CTAATACCTAAAGTTGAAAC	400	45	Konkel et al. (1999)
virB11	F - TCTTGTGAGTTGCCTTACCCCTTTT R - CCTGCGTGTCCTGTGTTATTTACCC	494	53	Datta et al. (2003)
iam	F - GCGCAAAATATTATCACCC R - TTCACGACTACTATGCGG	518	52	Carvalho et al. (2001)
racR	F - GATGATCCTGACTTTG R - TCTCCTATTTTTACCC	584	45	Datta et al. (2003)
pldA	F - AAGCTTATGCGTTTTT R - TATAAGGCTTTCTCCA	913	45	Datta et al. (2003)
cdtA	F - CCTTGTGATGCAAGCAATC R - ACACTCCATTTGCTTTCTG	370	49	Datta et al. (2003)
cdtB	F - CAGAAAGCAAATGGAGTGTT R - AGCTAAAAGCGGTGGAGTAT	620	51	Datta et al. (2003)
cdtC	F - CGATGAGTTAAAACAAAAAGATA R - TTGGCATTATAGAAAATACAGTT	182	47	Datta et al. (2003)

et al., 2002; Meinersmann et al., 1997). For PCR the conditions were as described above. The PCR products were visualized in gel electrophoresis, purified with Clean-Up Kit (A&A Biotechnology, Poland), and sequenced by Sanger sequencing (Macrogen Europe, The Netherlands). The forward and reverse sequences were assembled using Contig Express module in Vector NTI Express (Thermo Fisher Scientific, USA) and trimmed to 321 bp length covering the *flaA* SVR. The sequences were assigned *flaA*-SVR allele numbers according to the PubMLST database (http://pubmlst.org/campylobacter). The cluster analysis was performed using default parameters in MEGA X v. 10.1 (http://www.megasoftware.net). The maximum likelihood tree based on the *flaA*-SVR sequences was visualized in iTOL v4 (https://itol.embl.de).

The genetic diversity of Campylobacter isolates originating from poultry and humans was assessed by the Simpson's diversity index (ID) as described previously (Hunter and Gaston, 1988) using the online tool "Comparing Partitions" from the website http://www.comparingpartitions.info (Carriço et al., 2006).

2.4. Cell culture

The HeLa cell line used was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Penicillin and streptomycin (both at 100 IU ml^{-1}, Sigma Aldrich, USA) were added to protect against bacterial contamination. Incubation was conducted at 37 °C in the incubator with 5% CO_2 flow and 95% humidity. The whole medium from the culture vessel was pulled down every 24–48 h and the culture was supplemented with fresh medium. Cell monolayers were detached by addition of a trypsin – EDTA solution (0.05% (*w/v*) Trypsin and 0.02% (w/v) EDTA) (Sigma Aldrich, USA) and gentle tapping of the flask. Cell aggregates in the suspension were dispersed by sterile – pipetting before the cells were washed in fresh medium.

2.5. Cytotoxicity assay

The cell-free bacterial culture supernatants were used in the cytotoxicity assay. Isolates of *Campylobacter* spp. grown on mCCDA (Oxoid, UK) medium under microaerobic conditions at 37 °C for 48 h were suspended in PBS (phosphate buffered saline) and standardized spectrophotometrically to a concentration of 1×10^9 bacteria ml^{-1}. The concentration obtained was confirmed by counting the colonies grown on solid media prepared by successive culture of the subsequent decimal dilutions. After the concentration was standardized, 15% (*w/v*) of polymyxin B sulfate was added, in order to stimulate the release of cell-associated material. The suspension was incubated at 37 °C for 30 min and then centrifuged at 2500 $\times g$ for 20 min. The supernatant was passed through a 0.45 μm filter (Merk Millipore, USA), and the filtrate was spent directly for further study. Each *Campylobacter* spp. isolate was tested in duplicate in three separately performed cytotoxicity assays.

The cytotoxicity assays were performed according to the method described by Gilbert and Slavik (2004). Freshly trypsinized HeLa cells were suspended in flat bottom 96 – well plates at a density of 2×10^5 cells per well. After overnight incubation, previously prepared polymyxin B extracts were added to each well replacing 100 μl of growth medium. The plates were incubated for 24 h in a 5% CO_2 atmosphere at 37 °C. Then the MTT dye reduction assay was conducted in order to determine the percentage cell death by measuring the absorbance in each well at a wavelength of A_{540} with the use of Sunrise microtitre plate reader (Tekan, Switzerland). Cytotoxicity was calculated based on the formula given by Gilbert and Slavik (2004) and expressed as a percentage of cell death. Each *Campylobacter* isolate, based upon calculated HeLa cell death, was assigned a toxicity level: high (> 71% HeLa cell death), medium (31% - 70% HeLa cell death), low (1% - 30% HeLa cell death) and no toxic effect.

2.6. Adhesion and invasion assay

The adhesion and invasion assay was performed on HeLa cells according to the method described earlier by Konkel and Joens (1989) with slight modifications according to the protocol described previously (Wysok and Wojtacka, 2018). In order to conduct the experiment, HeLa cells were seeded into 24-well tissue culture plates (Nunc, Denmark) at a density of 5×10^4 cells per well and incubated at 37 °C in a humidified 5% CO_2 incubator until semi-confluent monolayers were obtained. HeLa cell monolayers were infected with approximately 1×10^7 cfu/ml of each *Campylobacter* isolate of human and poultry origin in duplicate. The plates were incubated for 3 h at 37 °C in a 5% CO_2 incubator. In order to kill all the extracellular bacteria MEM (Sigma Aldrich, USA) with gentamicin (250 μg/ml) was added to one of the duplicate wells. After incubation for a further 3 h the culture was lysed. The lysed monolayer suspensions were ten-fold diluted in PBS and spread onto mCCDA. After 24–48 h incubation under microaerophilic conditions the number of viable bacteria grown on plate was counted taking the dilution factor into account. The number of viable bacteria from the first well (with gentamicin) reflected the number of intracellular bacteria, *i.e.* the number of *Campylobacter* isolates able to invade HeLa cells. The second well (without gentamicin) represented the count of intracellular and extracellular bacteria combined. Adhesion was calculated by the formula [(cfu/ml from the second well at particular dilution – cfu/ml from the first well at particular dilution) x dilution ratio].

Each isolate was examined three times on separate occasions for adhesion and invasion.

2.7. Statistical analysis

The presence of virulence genes in different species and sources of the *Campylobacter* isolates was analyzed using contingency tables and Fisher's exact test. The cytotoxicity, adhesion and invasion levels were compared using the Mann – Whitney *U* test. Statistical significance was defined as $P < .05$.

3. Results

3.1. Isolation and identification of bacterial strains

Out of the 80 poultry carcasses, 50 (62.5%) were positive for *Campylobacter* spp. By PCR analysis 47 (94%) isolates were *C. jejuni* and 3 (6%) *C. coli*. In turn, among 1413 human stool samples evaluated, *Campylobacter* spp. were found in 54 (3.8%) samples, among which *C. jejuni* was detected in 49 (90.7%) and *C. coli* in 5 (9.3%) samples.

3.2. Diversity of isolates

flaA-SVR sequencing revealed a total of 28 different alleles among 50 *Campylobacter* isolates from poultry and 34 alleles among 54 isolates from humans (Fig. 1). The results showed considerable and equal diversity both in *Campylobacter* spp. isolates obtained from poultry and human with Simpson's diversity index of 0.973 (CI 95% 0.960–0.986) and 0.979 (CI 95% 0.967–0.991), respectively. The most commonly reported *flaA*-SVR alleles among poultry isolates were 11, 16, 36 and 320 covering 30% of all the isolates, respectively. These alleles were only observed among *C. jejuni* isolates. Similarly among isolates originating from humans the prevailing *flaA*-SVR sequence types were 16, 36 and 54 covering 22.2% of all the isolates, being observed only in *C. jejuni*. Altogether 22 *flaA*-SVR (48.9%) alleles out of a total of 45 alleles occurred in both poultry and human isolates, covering 20% and 22.2% of all human and poultry isolates respectively. Ten out of 28 (39.3%) and 12 out of 34 (50%) alleles from poultry and human isolates occurred only once.

3.3. Virulence genes

All *Campylobacter* strains of poultry origin, irrespective of the species, were positive for the three genes involved in adhesion (*flaA*, *cadF* and *racR*) (Table 2). Among genes related to invasion, the overall prevalence of *virB11*, *iam* and *pldA* genes were 22%, 84% and 100%, respectively. The species analysis showed the occurrence of statistically significant differences only for *virB11*. This marker was present in 22% of the *Campylobacter* spp. isolates (23.4% of *C. jejuni* and none of *C. coli* strains). The prevalence of the *cdtABC* gene cluster associated with cytolethal distending toxin production was confirmed in 96% of the *Campylobacter* isolates, however the detection rate was significantly lower in *C. coli* (33.3%) compared to *C. jejuni* (100%) ($P < .05$).

Among *Campylobacter* isolates obtained from humans all *C. jejuni* and *C. coli* isolates were positive for *flaA*, *cadF*, *racR* and *iam* (Table 2). The prevalence of *virB11* and *pldA* genes were 70.4% and 75.9%, however, the rates where insignificantly higher in *C. coli* (100% and 80%) compared to *C. jejuni* (67.3% and 75.5%) isolates. The *cdtABC* gene cluster was confirmed in 44 out of 49 (89.8%) of *C. jejuni* and in 2 out of 5 (40%) of *C. coli* isolates. Only *cdtA* was present in all isolates irrespective of species.

3.4. Cytotoxic activity

48 (96%) out of the 50 *Campylobacter* isolates obtained from poultry showed cytotoxicity at a mean level of 26.7% (Fig. 2a). *C. jejuni* isolates exhibited a slightly higher cytotoxicity (27.4%) compared to *C. coli* isolates (16.0%) ($P > .05$). The percentage of dead cells ranged between isolates from 1% to 57%. Analyzing the effect on HeLa cells, the majority of isolates (56%) showed low level of cytotoxicity, the remaining 44% and 4% of isolates demonstrated medium level cytotoxicity or no toxic effect, respectively (Fig. 3a).

In turn 51 out of 54 (94.4%) of *Campylobacter* isolates originating from humans in comparison to poultry isolates showed significantly higher cytotoxic activity ($P < .05$) at the level of 38.7% (values ranging from 7% to 77% of dead cells) (Fig. 2a). Cytotoxicity was observed in 48/49 (97.9%) *C. jejuni* and in 3/5 (60%) *C. coli* isolates at mean levels of 41.1% and 20.3%, respectively ($P < .05$). The majority (68.5%) of *Campylobacter* isolates showed a medium level of cytotoxicity towards HeLa cells. Whereas, 13 (24%), 1 (1.9%) and 3 (5.6%) out of 54 isolates exhibited low, high and no cytotoxicity, respectively (Fig. 3a). The association between cytotoxic activity and prevalence of genes involved in CDT production is shown in Fig. 4a. The dominant pattern observed both among poultry (in 48/50, 96%) and human (in 44/54, 81.5%) isolates was *cdtA_cdtB_cdtC*. The isolates manifesting this pattern showed higher cytotoxic activity in comparison to isolates with lack of certain *cdt* genes. The mean levels of cytotoxicity of isolates originating from poultry and human with confirmed presence of three adjacent *cdt* genes were 27.6% and 42.4%, respectively. Simultaneously two isolates (one human and one poultry isolate) despite the confirmed presence of *cdtA*, *cdtB* and *cdtC* genes showed lack of cytotoxic activity (Supplementary Table S1). The *Campylobacter* isolates with *cdtA_cdtC* pattern (noted among poultry and human isolates) and *cdtA_cdtB* (noted only among human isolates) showed lower levels of cytotoxicity (Fig. 4a). One isolate of poultry origin lacking all three genes and one isolate of human origin positive only for *cdtA*, didn't manifest any cytotoxic activity.

3.5. Adhesion and invasion

All *Campylobacter* isolates from poultry were able to adhere to epithelial cells at a mean level of 0.95% (ranging from 0.00011% to 7.3%) of the initial bacterial count (Fig. 2b). However, the majority of poultry isolates (42/50, 84%) adhered to HeLa cells from 0.00011% to up to 1% (Fig. 3b). Invasion was demonstrated in 36 out of 50 (72%) isolates at the mean level of 0.002% (ranging from 0.00007% to 0.06%) of starting viable inoculum (Fig. 2c). Mostly (19/36, 52.8%) poultry isolates exhibited the invasiveness in the range from 0.00007% to 0.001% (Fig. 3c). Species analysis showed higher adhesion abilities in *C. coli* (2.2%) in comparison to *C. jejuni* strains (0.9%) ($P > .05$). The opposite tendency was noted towards invasion abilities with *C. jejuni* (0.002%) in comparison to *C. coli* strains (0.0001%) ($P > .05$).

In relation to *Campylobacter* isolates from humans, adhesion was exhibited by 100% of the isolates at a mean level of 0.82%, and invasion was exhibited by 75.9% (41/54) of isolates at the mean level of 0.0005% of starting viable inoculum (Fig. 2b, c). The adhesion ranged from 0.001% to 7.7% with 46 out of 54 of isolates (85.2%) ranging from

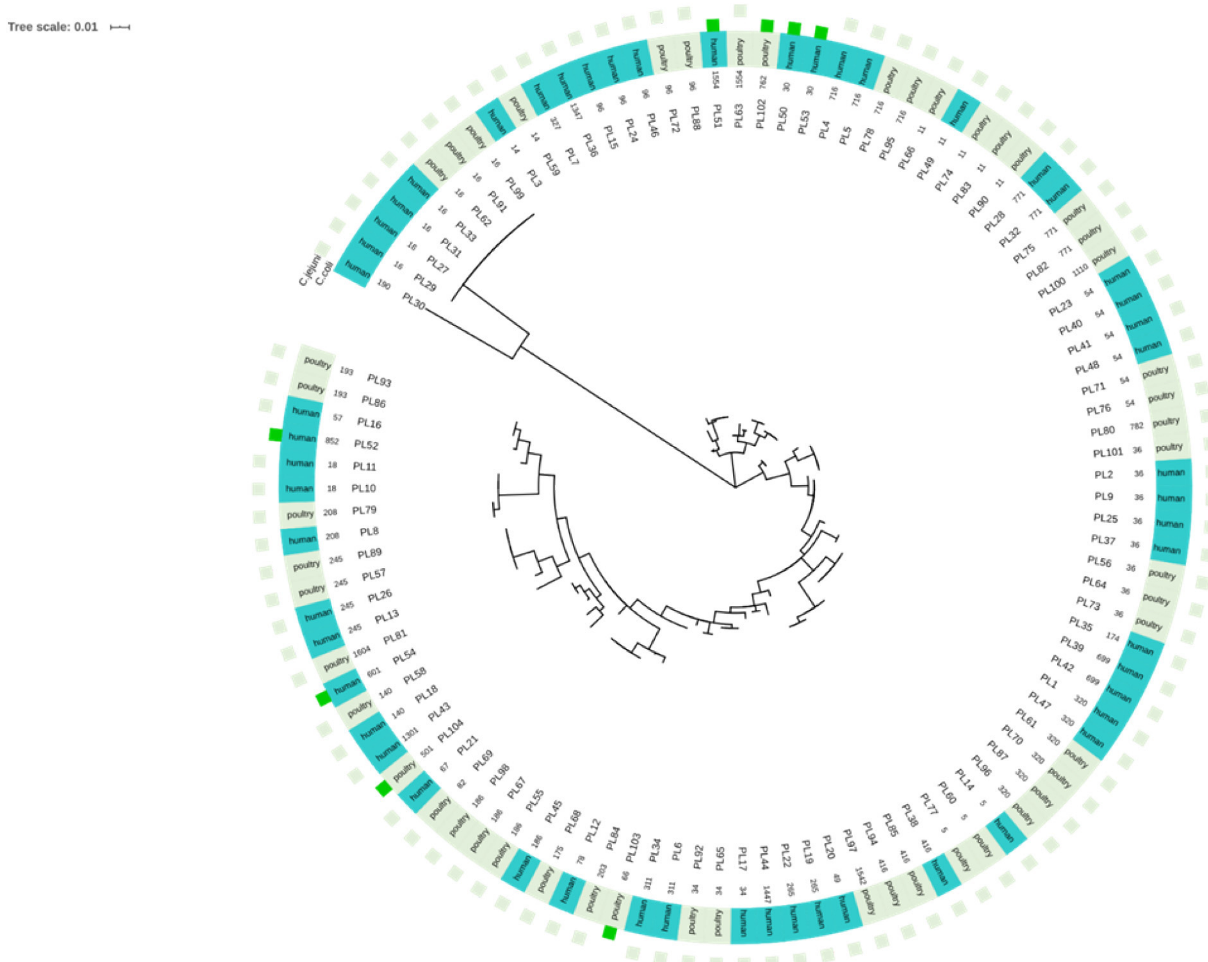

Fig. 1. Maximum likelihood tree of *Campylobacter flaA* SVR allele sequences among isolates originating from poultry and humans. For each isolate the following are shown: species, source and allele number. Visualized in the interactive Tree of life (iTol).

0.001% to 1% (Fig. 3b). Invasion ranged from 0.00003% to 0.002% with 35 out of 41 of isolates (85.4%) ranging from 0.00003% to 0.001% (Fig. 3c). Both adhesion and invasion was higher among *C. jejuni* (0.9% and 0.0005%) in comparison to *C. coli* (0.1% and 0.0001%) isolates, however the observed differences weren't statistically significant ($P > .05$).

The highest abilities to adhere to HeLa cells were observed in *flaA*-SVR allele 16 (in 4 out 7 isolates representing this type) and in allele 699 (in 2 out 2 isolates representing this type). Simultaneously only two isolates assigned to *flaA*-SVR allele 16 manifested also the highest abilities to invade epithelial cells (Supplementary Table S1) The 16 highly invasive isolates from poultry and humans represented 14 different *flaA* SVR alleles and thus did not present any single clone. In relation to adhesion, all *Campylobacter* isolates originating from poultry and humans, displayed only one gene pattern *flaA_cadF_racR* and were able to adhere to HeLa cells at similar level (Fig. 4b). In regard to invasion, the majority of human isolates (53.7%, 29/54) possessed all three tested genes (*virB11_iam_pldA*) and displayed the highest mean level of invasiveness (0.00049%) (Fig. 4c). The remaining 24.1% (13/54) and 16.7% (9/54) isolates possessing two genes (*virB11_iam* and

Table 2
The prevalence of virulence genes in *Campylobacter* isolates of poultry and human origin.

Source	Species	Adhesion			Invasion			CDT production			
		flaA	*cadF*	*racR*	*virB11*	*iam*	*pldA*	*cdtA*	*cdtB*	*cdtC*	*cdtABC*
Poultry	*C. jejuni* $n = 47$	47 (100%)	47 (100%)	47 (100%)	11 (23.4%)	40 (85.1%)	47 (100%)	47 (100%)	47 (100%)	47 (100%)	47 (100%)
	C. coli $n = 3$	3 (100%)	3 (100%)	3 (100%)	–	2 (66.7%)	3 (100%)	2 (66.7%)	1 (33.3%)	2 (66.7%)	1 (33.3%)
	Total $n = 50$	50 (100%)	50 (100%)	50 (100%)	11 (22%)	42 (84%)	50 (100%)	49 (98%)	48 (96%)	49 (98%)	48 (96%)
Human	*C. jejuni* $n = 49$	49 (100%)	49 (100%)	49 (100%)	33 (67.3%)	49 (100%)	37 (75.5%)	49 (100%)	44 (89.8%)	47 (95.9%)	44 (89.8%)
	C. coli n = 5	5 (100%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)	4 (80%)	5 (100%)	4 (80%)	2 (40%)	2 (40%)
	Total $n = 54$	54 (100%)	54 (100%)	54 (100%)	38 (70.4%)	54 (100%)	41 (75.9%)	54 (100%)	48 (88.9%)	49 (90.7%)	46 (85.2%)

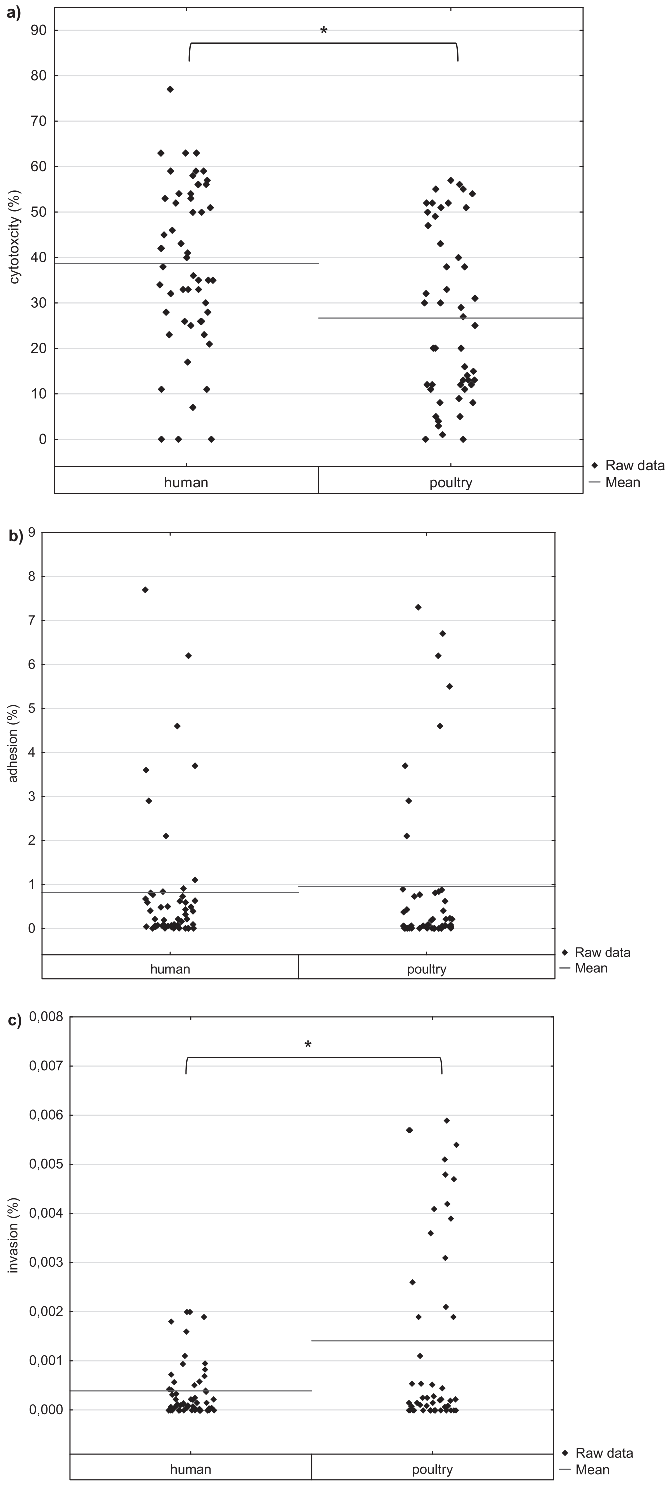

Fig. 2. The ability of *Campylobacter* isolates of human and poultry origin to produce cytotoxins (a), adhere to HeLa cells (b) and invade HeLa cells (c). Cytotoxicity was expressed as percent of dead cells. In relation to adhesion and invasion the data was plotted as percentage of the starting viable inoculum. Asterisks indicate the statistically significant differences between the mean level of pathogenic properties.

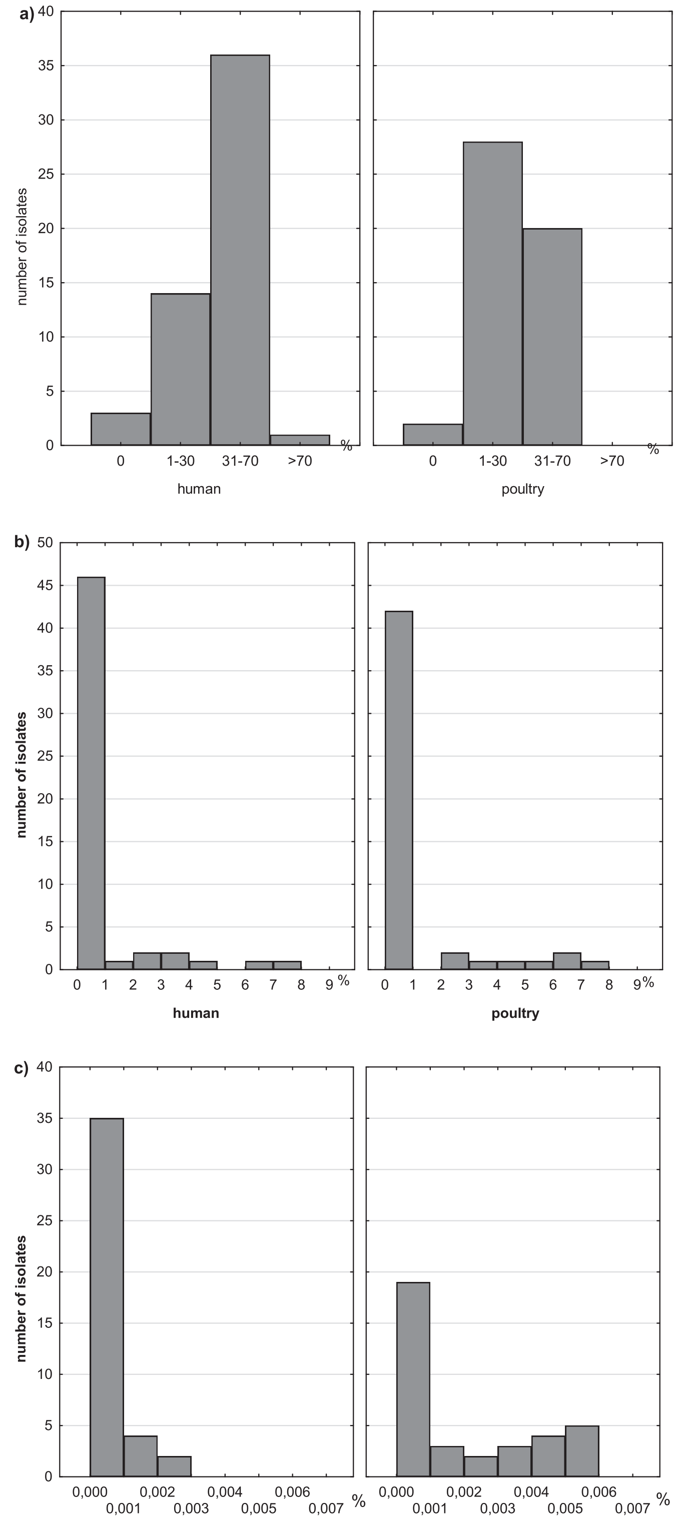

Fig. 3. Distribution of cytotoxic activity (a), adherence (b) and invasion (c) abilities in *Campylobacter* isolates of human and poultry origin. Cytotoxicity was expressed as percent of dead cells. In relation to adhesion and invasion the data was plotted as percentage of the starting viable inoculum.

iam_pldA, respectively), showed mean invasion level of 0.00041% and 0.00024% of starting viable inoculum ($P > .05$). The remaining 5.6% (3/54) of *Campylobacter* isolates from humans positive only for the *iam* gene, were not able to invade HeLa cells. In regards to isolates obtained from poultry, *iam_pldA* was the predominant gene pattern observed in 68% (34/50) of the isolates with invasiveness at a mean level of 0.0016%. The strongest invasion (0.002%) was noted in 16% (8/50) of isolates with confirmed prevalence of all three genes associated with invasion (*virB11_iam_pldA*). Poultry isolates manifesting gene patterns *virB11_iam_pldA* and *iam_pldA* displayed significantly higher ($P < .05$)

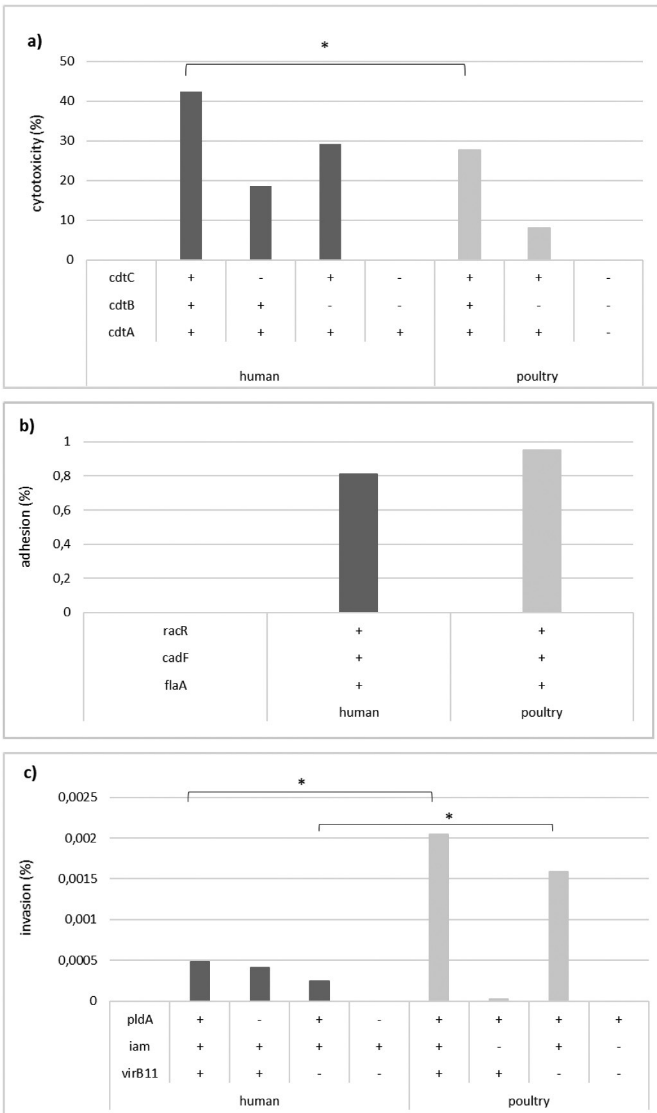

Fig. 4. Correlation between prevalence of pathogenic genes and the mean level of cytotoxic activity (a) and abilities to adhere to (b) and invade (c) HeLa cells in *Campylobacter* isolates of human and poultry origin. Cytotoxicity was expressed as percent of dead cells. In relation to adhesion and invasion abilities data were plotted as percentages of the starting viable inoculum. Asterisks indicate the statistically significant differences corresponding to the same gene pattern between isolates originating from different sources.

abilities to invade HeLa cells in comparison to human isolates. In turn the lowest invasiveness (0.00002%) was observed in isolates positive for *virB11_pldA*. The isolates only positive for *pldA* were not able to invade HeLa cells.

4. Discussion

Campylobacteriosis is the most commonly reported bacterial food-borne disease in both industrialized and developing countries. Poultry is considered as the main source of campylobacteriosis in humans, however these bacteria colonize the digestive track of numerous other species, including domestic farm animals, dogs, cats, various wildlife, and rodents (Horrocks et al., 2009). Our findings confirmed the common prevalence of *Campylobacter* spp. in poultry carcasses (62.5% positive samples). This rate is lower than that registered in the Netherlands (> 82%) and the United Kingdom (76%), but higher than in Sweden and Norway (27% and 18%, respectively) (Hein et al., 2003). The implementation of the process hygiene criterion for *Campylobacter* in broiler carcasses in EU in 2018 is likely to reduce the contamination of poultry by *Campylobacter* in the future, however, this remains to be seen. Because the infectious dose is low the consumption of undercooked poultry and poultry products or ready-to-eat foods that have been in contact with raw chicken leads easily to human infection (Marotta et al., 2015; Kaakoush et al., 2015). In this study the overall prevalence of campylobacter in humans diagnosed with diarrhea was 3.8%. A similar isolation rate was noted by Meistere et al. (2019) in Latvia (5.3%), however higher rates were demonstrated in Algeria (17.7%), Tanzania (18.0%) and Bangladesh (17.4%) (Coker et al., 2002). Our results indicated, that both among poultry and human isolates, *C. jejuni* was the dominant species. Many authors underlined, that poultry is predominantly (up to 95%) colonized by *C. jejuni*, less often with *C. coli* and rarely with other *Campylobacter* species (Hodges et al., 2019; Meistere et al., 2019; Wieczorek and Osek, 2005). Similarly campylobacteriosis in humans in most cases (80–90%) is caused by *C. jejuni*, while 7% by *C. coli* and the remainder by *C. lari, C. hyointestinalis, C. upsaliensis* (Jain et al., 2008; Nadeau et al., 2003).

Diversity of the *Campylobacter* isolates was specified based on sequencing the SVR of the *flaA* gene. In this study a total of 45 *flaA*-SVR alleles were identified among 104 *Campylobacter* isolates of human and poultry origin. Similar high levels of genetic diversity of *Campylobacter* isolates, using this method, were observed in Spain, Norway and Iceland by Wassenaar et al. (2009), in Brazil by Gomes et al. (2016) and in Italy by Marotta et al. (2015). In a previous study conducted by Wieczorek et al. (2019) in Poland *flaA*-SVR alleles 16, 54, 36, 34 and 287 prevailed among both human and poultry isolates. In our study four out of these five genotypes were also identified (Giacomelli et al., 2012; Morais-Rios et al., 2018; Nadeau et al., 2003; Zheng et al., 2006), both among human and poultry isolates, and these alleles were the most commonly identified ones. Previously *e.g. flaA*-SVR allele 36 was also common in Norway, Iceland and Spain (Wassenaar et al., 2009), but missing in Brazil (Gomes et al., 2016). However in Brazil *flaA*-SVR allele 34 was predominant (Di Giannatale et al., 2019), similar to Spain, but not noted in Norway and Iceland (Wassenaar et al., 2009). These results are in line with the previous study by Wassenaar et al. (2009) which showed that the differences between geographical regions exceeded those observed in the distribution of *Campylobacter* genotypes among different hosts isolated in the same country. Comparing the diversity of *Campylobacter* isolates, the total number of *flaA*-SVR alleles was higher in human than poultry isolates. Out of 28 different alleles noted in poultry, 17 (60.7%) were observed in human isolates (covering 59.3% of human isolates), suggesting poultry as an important source of *Campylobacter* infection.

Identifying pathogenicity factors is very important in order to better understand the risk of campylobacteriosis associated with different strains. However, the prevalence of these factors does not directly determine the strains virulence, and the detection of genes associated with pathogenicity should be complemented by gene expression analysis and testing in a disease model. In the present study, all *Campylobacter* isolates, irrespective of the source, possessed the three examined genes (*flaA*, *cadF*, *racR*) encoding proteins involved in adhesion. These virulence factors have also previously been shown to be common in poultry and human clinical isolates in both Poland (Wieczorek and Osek, 2008) and different geographical regions (Datta et al., 2003; Talukder et al., 2008). However, Datta et al. (2003) underlined that the occurrence of *racR* depended on the source. These authors found *racR* in 100% of poultry and 91.1% of human isolates, which is in accordance with our findings. Whereas Rizal et al. (2010) observed the prevalence of this gene only in 5.5% of human and 18.33% of poultry isolates. In order to assess the capacity of the *Campylobacter* isolates to adhere to the human intestinal epithelium the cell culture model was used. We noted adhesion in all *Campylobacter* isolates, regardless of the source. The level of adhesion of the poultry and human isolates didn't vary considerably (observed mean adherences 0.95% and 0.82%, respectively). Similarly adhesion was demonstrated by Nadeau et al. (2003) in regard to poultry and human isolates, as well as by Zheng et al. (2006) in isolates from retail raw chicken, turkey, beef and pork. However, their maximal values were considerably lower than the ones observed in this study. Zheng et al. (2006) reported the highest adherence at the level 3.115% of the starting viable inoculum, while in this study the maximal values reached up to 7.7%, and adhesion > 3.115% was shown by 12% of poultry and 9.3% of human isolates. The studies conducted by Nadeau et al. (2003) showed even lower (1.8% of the bacterial inoculum) numbers of bacteria that adhered to epithelial cells. Simultaneously, we noted, that 16% and 13% of poultry and human isolates adhered to epithelial cells > 1.8%. Knudsen et al. (2006) and Pielsticker et al. (2012) emphasized, that the outcome of *Campylobacter* colonization patterns can be influenced by strain variations. Therefore further studies are needed to determine if adhesion abilities are associated with specific lineages of *Campylobacter* as suggested by our results.

The ability to invade the epithelial cells of the gastrointestinal tract is also crucial for the development of *Campylobacter* infection in humans (Zheng et al., 2006). From several genes involved in host cell invasion we determined the occurrence of the *virB11*, *iam* and *pldA* genes. The *pldA* gene was present in 100% of poultry and 75.9% of human isolates. High prevalence of this virulence marker was noted also in previous studies both in isolates of human (Biswas et al., 2011; Datta et al., 2003; Talukder et al., 2008) and poultry origin (Datta et al., 2003). Another marker associated with invasion, *iam* gene, was detected in 84% and 100% of poultry and human isolates, respectively. These findings are in accordance with studies conducted by Biswas et al. (2011), who noted this marker in 92.31% of human clinical isolates. Slightly lower prevalence of 77.77% in poultry and 60% in human isolates were shown by Rizal et al. (2010). In contrast, Rożynek et al. (2005) found *iam* gene only in 20% of *Campylobacter* isolates from children with diarrhea, while the same was observed by Carvalho et al. (2001) in asymptomatic patients. These findings suggest that it may not be a universal marker of the severity of *Campylobacter* infection (Rożynek et al., 2005), however further studies are needed to confirm the findings. Interestingly *virB11* was noted in 22% of poultry and 70.4% of human isolates. In previous studies, this marker was identified only sporadically in human isolates, in 3.6% in Chile (González-Hein et al., 2013), 5.77% in Canada (Biswas et al., 2011) and 17% in Poland (Tracz et al., 2005), while the prevalence among poultry isolates ranged from 9.5% in Japan (Datta et al., 2003) to 66% in Poland (Wieczorek and Osek, 2008). Furthermore, the 22% of poultry and 70.4% of human isolates were positive for all three tested genes involved in invasion (*pldA*, *iam* and *virB11)* and showed invasion using HeLa cells *in vitro*. Also isolates lacking only one gene (with gene patterns *virB11_pldA* or *iam_pldA*) were invasive. However the isolates with the *iam_pldA* gene pattern exhibited invasiveness similar to those noted in isolates with *pldA_iam_virB11* gene pattern both in poultry (0.002% and 0.0015%) and human isolates (0.0027% and 0.0019%). Significantly different invasion was seen only between *Campylobacter* isolates with the presence of all examined genes and with the lack of *iam* gene both in poultry (0.002% and 0.000023%) and human isolates (0.0027% and 0.00007%). Our study thus suggests that the presence of *iam* might have a significant impact on the strains ability to invade epithelial cells. Further studies are needed to confirm this findings and to reveal which other genetic markers may play a role in invasiveness.

Zheng et al. (2006) suggested, that adherence of *Campylobacter* might facilitate invasion into host cells but did not necessarily lead to invasion. In this study not all isolates exhibited positive correlation between adhesion and invasion. Invasiveness was observed in 72% of poultry at a mean level of 0.002 and in 75.9% of human isolates at a mean level of 0.0005% of starting bacterial inoculum. Similar results were noted by Nadeau et al. (2003), the percentage of poultry and human isolates showing invasive properties were 63.6% and 58.3% at levels ranging from 0.0001% to 0.005% of the bacterial inoculum. Simultaneously, these authors showed, that 21.3% of the isolates represented high invasiveness (above 0.002%). In our study 15.4% of all isolates were highly invasive (> 0.002%) and this was more common among poultry (28%, 14/50) than human (3.7%, 2 out of 54) isolates.

The ability to produce toxins is one of the crucial virulence factors involved in the course of *Campylobacter* infection. Among toxins formed by *Campylobacter* the cytolethal distending toxin (CDT) is best recognized. Activity of CDT requires the expression of three adjacent genes *cdtA*, *cdtB* and *cdtC* (Bang et al., 2001). The results of numerous studies indicate high prevalence of *cdtABC* genes in *Campylobacter* strains regardless of the source of isolation (de Carvalho et al., 2013; Findik et al., 2011), which is in line with our findings demonstrating the presence of the three *cdt* genes in 96% of poultry and 85.2% of human isolates. Recent studies analyzing the distribution of *cdtA*, *cdtB* and *cdtC* genes separately or together in the *cdtABC* cluster in C. *jejuni* and C. *coli* indicate that their prevalence in isolates from poultry and other sources exceeds 90% (Bang et al., 2001; Datta et al., 2003). However our study revealed the occurrence of significant difference in the detection rates of *cdtABC* genes in *C. jejuni* and *C. coli* isolates originating from poultry (100% and 33.3%) and human (89.8% and 40%). These observations were also noted by Rożynek et al. (2005), who noted lower frequency of the *cdt* operon in *C. coli* than *C. jejuni* isolates both in poultry (87.2% and 100%) and human origin isolates (5.6% and 98%). Also Van Deun et al. (2007), in a study on *Campylobacter* strains from different sources, noted that not all *Campylobacter* strains produced cytotoxin and more pronounced CDT production was associated with human strains. According to Gilbert and Slavik (2004), although toxicity is described as a primary determinant of *Campylobacter* pathogenicity, a considerable percentage of *Campylobacter* isolates found in the food chain may not possess toxicity levels high enough to cause disease in humans. In this study high toxic effect was noted in 20% of poultry isolates and 37% of human isolates ($P < .05$). Simultaneously low level of cytotoxicity was noted in 42% and 7.4% of poultry and human isolates ($P < .05$), respectively. Our findings are in line with the study conducted by Gilbert and Slavik (2004), who confirmed the prevalence of statistically significant differences in toxicity levels between isolates obtained from humans suffering from campylobacteriosis and isolates obtained from chicken carcasses. These authors noticed that there were no human isolates manifesting low cytotoxicity level, while 56% of poultry isolates obtained from postchilled chicken carcasses were assigned to this group. Moreover, Ripabelli et al. (2010) underlined the occurrence of differences in toxicity level among *Campylobacter* species noting weak toxic capabilities most commonly among *C. coli* strains. This result is in accordance with our findings, as no cytotoxic effect was shown in 33.3% of *C. coli* and only 2.1% of *C. jejuni* isolates recovered from poultry, while for human isolates the values observed were 40% and 2.1%, respectively. Simultaneously these isolates displaying no toxic effect manifested the lack of all or two genes involved in cytolethal distending toxin production.

Campylobacter spp. from poultry should always be considered as potential pathogens for humans. Both poultry and human isolates were shown to carry genes determining pathogenicity of *Campylobacter* and identical *flaA*-SVR alleles. Interestingly, isolates recovered from poultry carcasses manifested higher invasion abilities when compared to human isolates. Our results underlined the importance of the *iam* gene, since all *iam* – positive isolates were associated with a higher level of invasion. Furthermore, poultry isolates with a similar gene pattern to the human isolates showed even higher levels of invasion suggesting that other genetic traits, than the ones already identified, may further govern this ability. In relation to cytotoxicity, significantly higher cytotoxic capabilities were observed for *Campylobacter* isolates from humans compared to chicken carcasses, and cytotoxicity was clearly associated with the presence of at least two genes from the *cdtABC* operon. Furthermore, the *cdtABC* operon was more common in *C. jejuni* compared to *C. coli*, and a similar trend was seen in cytotoxicity *in vitro*. It remains to be shown how cytolethal distending toxin is expressed during infection in different hosts and how it relates to the disease outcome.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical committee

The human *Campylobacter* isolates were obtained from the microbiological laboratory of the regional sanitary-epidemiological units after obtaining permission of the chair of the units.

Declaration of competing interest

The authors have no conflict of interest to declare.

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